AN EXPERIMENT IN AUTOTROPHIC FERMENTATION

Microbial Oxidation of Hydrogen Sulfide

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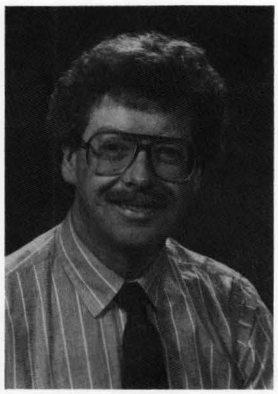
THERE HAS BEEN AN increasing effort in recent years to introduce chemical engineering students to biological processes. These efforts may be rather modest, with the incorporation of one or more experiments in enzyme kinetics or fermentation into the undergraduate laboratory, or more broadly based with formal course work in microbiology, biochemistry and biochemical engineering, and blocks of time in the laboratory devoted to bioengineering experiments. However, even in the more comprehensive programs the chemical engineering student is typically exposed to only one basic type of fermentation, that which is based on heterotrophic metabolism. In other words, the microorganisms which make up the fermentation culture utilize *organic* compounds as carbon sources and light or the oxidation of organic compounds as a source of energy. Another way of life exists by which microorganisms may derive both carbon and energy from inorganic sources. This is termed autotrophic metabolism. Autotrophic organisms are becoming increasingly important commercially in waste treatment, coal and sour gas desulfurization, and mineral leaching applications. This experiment is designed to introduce the student to fermentation based on autotrophic metabolism. The subject of substrate inhibition is also addressed. This experiment is not recommended as a first introduction to fermentation but as a demonstration of the wide range of metabolic capabilities of microorganisms.

Specifically this experiment utilizes the autotrophic bacterium *Thiobacillus denitrificans* to anaerobically oxidize $H_2S(g)$ to sulfate in a batch stirred tank reactor. Hydrogen sulfide is shown to be an inhibitory substrate for the bacterium; however, under sulfide-limiting conditions rapid and complete oxidation of H_2S is observed with undetectable levels of H_2S

TABLE 1
Medium for Anaerobic Growth of *Thiobacillus denitrificans*

COMPONENT	PER LITER
Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgSO_4 \cdot 7H_2O$	0.4 g
NH_4Cl	0.5 g
$CaCl_2$	0.03 g
$MnSO_4$	0.02 g
$FeCl_3$	0.02 g
$NaHCO_3$	1.0 g
KNO_3	5.0 g
$Na_2S_2O_3$ or $H_2S(g)$	10.0 g (thiosulfate)
Trace metal solution*	15.0 ml
Mineral water	50.0 ml

* See reference (2).



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in the bioreactor outlet gas. Provided the bioreactor can be sampled periodically over 1-2 days, the stoichiometry of the reaction is readily determined.

BACKGROUND

In nature there exists a large and widely distributed group of microorganisms which play a central role in the maintenance of the carbon, nitrogen, and sulfur cycles. These microorganisms are termed lithotrophic if they are capable of deriving energy and/or reducing equivalents from the oxidation of inorganic compounds. Chemolithotrophs derive both energy and reducing equivalents from such reactions, while photolithotrophs derive energy from the absorption of radiant energy and reducing equivalents from oxidation of inorganic compounds. Those microorganisms capable of also deriving carbon for biosynthesis from an inorganic source (carbon dioxide) are termed autotrophic. Examples of inorganic energy sources for chemoautotrophs include hydrogen, ammonia, iron (II) salts, elemental sulfur, thiosulfates, and sulfides. The interrelationship between substrate oxidation and biosynthesis in chemoautotrophic organisms is illustrated in Figure 1.

Thiobacillus denitrificans is a strict autotroph and facultative anaerobe first described in detail by Baalsrud and Baalsrud [1]. Thiosulfate, elemental sulfur, and soluble sulfide may be utilized as energy sources with oxidation to sulfate. Under anaerobic conditions nitrate may be used as a terminal electron acceptor with reduction to elemental nitrogen.

Anaerobic growth of *T. denitrificans* on H_2S(g) has been described in detail by Sublette and Sylvester [2-4]. The medium used to grow *T. denitrificans* is given in Table 1. When H_2S served as the energy source it was bubbled into the reactor at a rate sufficiently low as to maintain sulfide-limiting conditions. In other words, H_2S was introduced into the cultures at a rate which was less than the maximum oxidation capacity

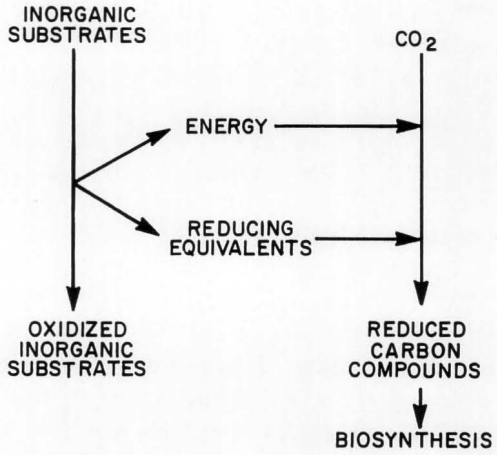

FIGURE 1. Chemoautotrophic metabolism

of the biomass. Stock cultures and start-up cultures used thiosulfate as an energy source. Under anaerobic conditions nitrate was the terminal electron acceptor. Bicarbonate was the carbon source and ammonium the source of reduced nitrogen. The medium also contained a phosphate buffer and sources of various essential mineral nutrients.

Sublette and Sylvester [2, 3] reported that when H_2S(g) was introduced into anaerobic cultures of *T. denitrificans* previously grown on thiosulfate, the H_2S was immediately metabolized with no apparent lag. Typically the feed gas contained about one mole percent H_2S. Initial loadings were in the range of 4-5 mmoles H_2S/hr-g biomass. With sufficient agitation to reduce the average bubble diameter to about 0.25 cm and gas-liquid contact times of 1-2 sec, H_2S was undetectable in the reactor outlet gas by GC/MS. Less than 1 μM of total sulfide (H_2S, HS^-, S^{-2}) was observed in the reactor medium during periods of up to thirty-six hours of batch operation. No intermediates of sulfide oxidation (elemental sulfur or sulfite) were detected; however, sulfate accumulated in the reactor medium as H_2S was removed from the feed gas. Oxidation of H_2S to sulfate was accompanied by growth as indicated by an increase in optical density and protein concentration and a decrease in the NH_4^+ concentration in the medium. The reaction was acid producing requiring hydroxide (OH^-) equivalents to be pumped into the reactor to maintain an optimum pH of 7.0. Small amounts (< 40 μM) of nitrous oxide, N_2O, could be detected in the reactor outlet gas. However, no other intermediates of nitrate reduction were observed to accumulate while nitrate was consumed. The stoichiometry of anaerobic H_2S oxidation by *T. denitrificans* in batch reactors as reported by Sublette and Sylvester [2] is given in Table 2.

Sublette and Sylvester [2] also reported that H_2S

TABLE 2
Stoichiometry of Anaerobic H_2S Oxidation by *T. denitrificans* in Batch Reactors*

SO_4^{-2}/H_2S	1.04	mole/mole
NO_3^-/H_2S	1.36	mole/mole
NH_4^+/H_2S	0.12	mole/mole
OH^-/H_2S	1.60	equivalents/mole
Biomass/H_2S	12.1	g/mole

* Average of four determinations

is an inhibitory substrate for *T. denitrificans*. Inhibitory effects were observed at total sulfide concentrations as low as 200 μM with total inhibition observed at 1000 μM. The total sulfide concentration in the medium of batch anaerobic *T. denitrificans* reactors operated under sulfide-limiting conditions was well below inhibitory levels. However, these authors reported that if the maximum capacity of the biomass for H_2S oxidation was exceeded, inhibitory levels of sulfide quickly accumulated in the medium. This upset condition was characterized by H_2S breakthrough in the outlet gas, release of large amounts of N_2O and accumulation of elemental sulfur in the medium. It was observed that this upset condition was reversible if the cultures were not exposed to the accumulated sulfide for more than two to three hours. Reduction in the H_2S feed rate following an upset condition reduced the H_2S and N_2O concentrations in the outlet gas to pre-upset levels with elemental sulfur oxidized to sulfate. The H_2S loading at which the specific activity of the *T. denitrificans* biomass was exceeded resulting in upset was observed to be 5.4-7.6 mmoles H_2S/hr-g biomass under anaerobic conditions.

The autotrophic medium described in Table 1 will not support the growth of heterotrophs since there is no organic carbon source. However, Sublette and Sylvester [4] observed that if aseptic conditions were not maintained a heterotrophic contamination developed in a *T. denitrificans* culture growing on thiosulfate or H_2S. Evidently *T. denitrificans* releases organic material into the medium in the normal course of growth or through lysis of nonviable cells which supports the growth of heterotrophs. Sublette and Sylvester reported that the heterotrophic contamination had no discernable effect on H_2S metabolism by *T. denitrificans*.

EXPERIMENTAL PROCEDURE

Samples of *Thiobacillus denitrificans* may be obtained from the American Type Culture Collection, Rockville, Maryland, or from the author. Stock cultures may be grown anaerobically on thiosulfate in 10 ml culture tubes at 30°C. When dense qrowth appears (three to four days with fresh inoculum) store at 4°C until used. Stocks should be transferred every thirty to sixty days to maintain vigorous cultures. Stock cultures do not need to be grown aseptically. Figure 2 presents a schematic diagram of the equipment required to culture *T. denitrificans* anaerobically on H_2S(g).

An investigation of the anaerobic oxidation of H_2S by *T. denitrificans* is described below. Details of the analytical methods required for a thorough study of the stoichiometry of the process are also presented. It is intended that the experiment can be used to introduce the subjects of autotrophic fermentation and substrate inhibition at a number of levels of difficulty and challenge to the students. A straightforward demonstration of the detoxification of a hazardous material by a bacterium can be conducted requiring less than three hours of student participation. However, an investigation of the stoichiometry of the process would require intermittent observation and sampling of H_2S cultures for one to two days plus time for sample analysis.

Before describing the experimental protocol a word of caution is in order. Hydrogen sulfide is a highly toxic gas. The threshold limit value (time weighted average) for H_2S exposure is 10 ppm (7-8 hrs). The threshold limit value for short term exposure is 15 ppm (15 minutes). It is therefore necessary that the laboratory instructor carefully monitor all operations involving H_2S and, as indicated in Figure 2, the entire fermentation system *must* be located in a fume hood.

Anaerobic Oxidation of H_2S

A working culture of *T. denitrificans* may be developed by growing on thiosulfate at 30°C and pH 7.0 using the medium described in Table 1. The purpose of this prior cultivation on thiosulfate is to develop a sufficient concentration of biomass in the reactor so that an appreciable rate of H_2S can be fed to the reactor without exceeding the biooxidation capabilities of the biomass. Otherwise sulfide would accumulate in the reactor medium to toxic levels.

The recommended culture vessel (see Figure 2) is a 1-2 1 jacketed beaker with a large silicone rubber

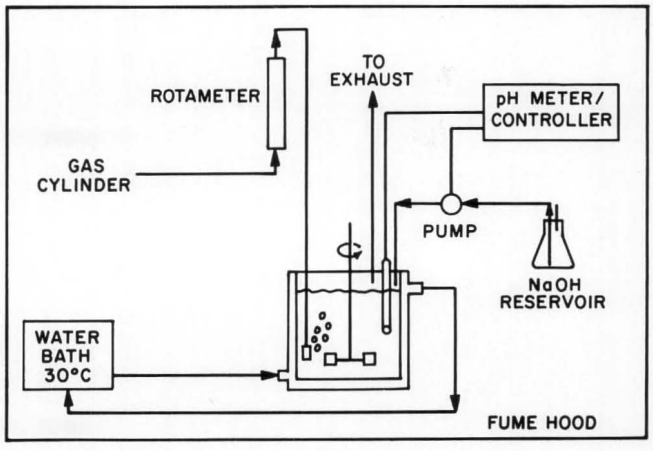

FIGURE 2. Schematic diagram of equipment required to culture* T. denitrificans *anaerobically on H_2S(g)

Chemolithotrophs derive both energy and reducing equivalents from such reactions, while photolithotrophs derive energy from the absorption of radiant energy and reducing equivalents from oxidation of inorganic compounds. Those microoganisms capable of also deriving carbon for biosynthesis from an inorganic source (carbon dioxide) are termed autotrophic.

stopper to support probes and inlet and outlet ports. Temperature may be controlled by circulating water at 30°C through the jacket of the reactor. The pH may be monitored and controlled by a pH meter/controller which activates a peristaltic pump to deliver 6 N NaOH to neutralize acid produced by the biooxidation of thiosulfate or H_2S. A pH stability of ± 0.2 units is desirable. If the controller also activates a laboratory timer, the rate of NaOH addition can be monitored. A gas feed of 5 mole % CO_2 in nitrogen at 30 ml/min is recommended during growth on thiosulfate to ensure the continuous availability of a carbon source. Gas mixtures are fed to the reactors from cylinders of compressed gas through two stage stainless steel regulators and rotameters. Gas is introduced into the culture medium by means of a glass fritted sparger. A four or six bladed, flat disk impeller may be used for agitation at 200-300 rpm to produce good gas-liquid contacting.

As noted previously, heterotrophic contamination has little or no effect on the growth of *T. denitrificans* in an autotrophic medium. Therefore, it is not necessary to sterilize the reactor or any associated equipment.

When 1-2 l of thiosulfate medium is inoculated with 20 ml of a fresh stock culture, a working culture is produced in approximately sixty hours. The medium described in Table 1 is nitrate limiting; therefore, when the nitrate is depleted the culture stops growing. At this point the culture will have an optical density at 460 nm of about 1.0.

The pathways for sulfide and thiosulfate oxidation to sulfate in *T. denitrificans* are not independent but have two common intermediates [5]. In the presence of thiosulfate the rate of sulfide oxidation would be reduced because of competition between intermediates of thiosulfate and sulfide oxidation for the same enzymes of the sulfur pathway. Therefore, prior to the introduction of H_2S to *T. denitrificans* cultures, residual thiosulfate must be removed. This may be accomplished by sedimenting the cells by centrifugation at 4900 × g for ten minutes at 25°C. A refrigerated centrifuge is preferred. However, any centrifuge is acceptable if the temperature of the cell suspension does not exceed 45°C during harvesting. The supernatant is discarded and the cells resuspended in growth medium without thiosulfate. Following a second centrifugation the washed cells are resuspended in medium without thiosulfate and transferred back to the reactor. It is recommended that the reactor and all probes be rinsed in distilled water to remove residual thiosulfate prior to reintroduction of cells.

Once thiosulfate has been eliminated, hydrogen sulfide can now be introduced into the culture by changing the feed gas to include H_2S. A composition of 1 mole % H_2S, 5 mole % CO_2 and balance N_2 is recommended. If the OD_{460} of the culture is at least 0.8, a feed rate of about 50 ml/min will not exceed the biooxidation capabilities of the culture. It is recommended that the H_2S feed rate be brought to this level stepwise over about thirty minutes. With proper agitation to achieve good gas-liquid contacting, H_2S will be undetectable in the gas outlet of the reactor.

The stoichiometry of anaerobic oxidation of $H_2S(g)$ by *T. denitrificans* can be obtained by sampling the reactor contents over a period of 24-48 hours as H_2S is removed from the feed gas. Of particular interest would be the concentrations of sulfate (SO_4^{-2}), nitrate (NO_3^-), ammonium ion (NH_4^+), elemental sulfur and biomass in the reactor medium. Analytical methods are discussed below.

The inhibitory nature of H_2S as a substrate can be demonstrated by increasing the H_2S feed rate stepwise until H_2S breakthrough is seen. When breakthrough occurs, nitrous oxide (N_2O) will also be detected in the outlet gas and elemental sulfur seen to accumulate in the reactor medium. Sulfur will give the medium a milky white color. As described in a previous section, the upset condition is reversible if not prolonged. However, if the upset condition is allowed to persist the outlet H_2S concentration becomes equal to the inlet, indicating complete loss of biooxidation activity in the culture.

Analytical

Feed gas and reactor outlet gas may be analyzed for H_2S and N_2O by gas chromatography. Using a thermal conductivity detector the detection limit for H_2S is about 2-4 μM with a 0.25 ml sample. In our laboratory a 10-ft by 1/8-in ID Teflon column containing Porapak QS (Waters Associates) has been used with a helium flow rate of 20 ml/min. A column temperature of 70°C and injector and detector temperatures of 200°C are satisfactory. Under these conditions the retention times of N_2, CO_2, N_2O, and H_2S are 0.8, 1.8,

2.2, and 5.3 minutes, respectively. H_2S is quantitated by comparing chromatograms of samples to chromatograms produced by a certified primary standard (Matheson Gas Co.). If a gas chromatograph is unavailable, H_2S and N_2O may also be determined to ± 25% using Gastec Analyzer Tubes (Yokohama, Japan).

Nitrate may be determined in thiosulfate free samples by the cadmium reduction method [6]. Ammonium ion may be determined by the Nessler method without distillation [6]. Sulfate is readily determined turbidometrically [6]. Premeasured reagents for these analyses may be purchased from Hach Chemical Co.

Thiosulfate may be determined by titration with standard iodine solution using a starch indicator [7]. Elemental sulfur may be collected by filtration on 0.45 micron Millipore Type HA filters and determined by reaction with cyanide to produce thiocyanate. Thiocyanate may be quantitated as $Fe(SCN)_6^{-3}$ which has a molar extinction coefficient in water at 450 nm of $3.37 \times 10^3\ M^{-1}\ cm^{-1}$ [8].

Biomass may be determined in terms of whole cell protein by sonication followed by colorimetric analysis by the micro modification of the Folin-Ciocalteau method [9, 10]. (Folin-Ciocalteau reagent may be purchased from Anderson Laboratories.) Cells are suspended in 10-20 ml of 20 mM phosphate buffer, pH 7.0, and sonicated with a sonic probe until the suspension is clarified. In our laboratory a Braun-Sonic 1510 with a 3/4 in. probe is used at 150 watts for two three-minute periods with intermittent cooling. The resulting protein solution is analyzed directly without further treatment. Bovine serum albumin (Sigma Chemical Co.) is used as a standard. The protein content of *T. denitrificans* cells grown on $H_2S(g)$ is 60 ± 3% [2]. Using this figure the results of protein analyses may be converted to dry weight *T. denitrificans* biomass.

SAMPLE RESULTS

In a typical batch experiment the oxidation of 18.3 mmoles of H_2S was accompanied by the production of 18.8 mmoles sulfate and 246 mg of biomass. A total of 27.0 mmoles nitrate, 2.2 mmoles ammonium ion and 31.8 meq of hydroxide ion were utilized.

Figures 3a and 3b summarize the results of analysis of the medium of an anaerobic reactor as H_2S is oxidized by the culture. Sulfate is seen to accumulate in the medium. The concentration of biomass increases as the cells grow using H_2S as an energy source and correspondingly the optical density increases with time. Nutrient levels (NO_3^- and NH_4^+) decline as H_2S

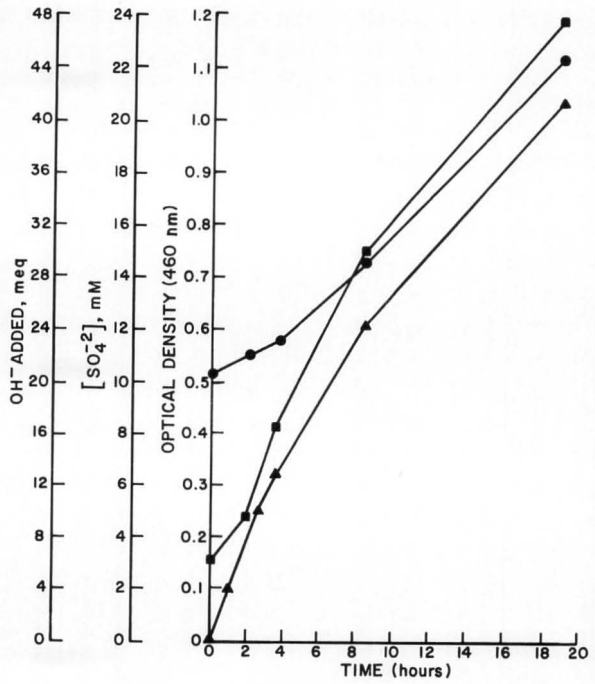

FIGURE 3a. Optical density, concentration of sulfate (SO_4^{-2}) and hydroxide ion (OH^-) utilized in an anaerobic* T. denitrificans *batch reactor receiving 1.25 mmoles/hr hydrogen sulfide (H_2S) feed. OD (●); SO_4^{-2} (■); OH^- (▲).

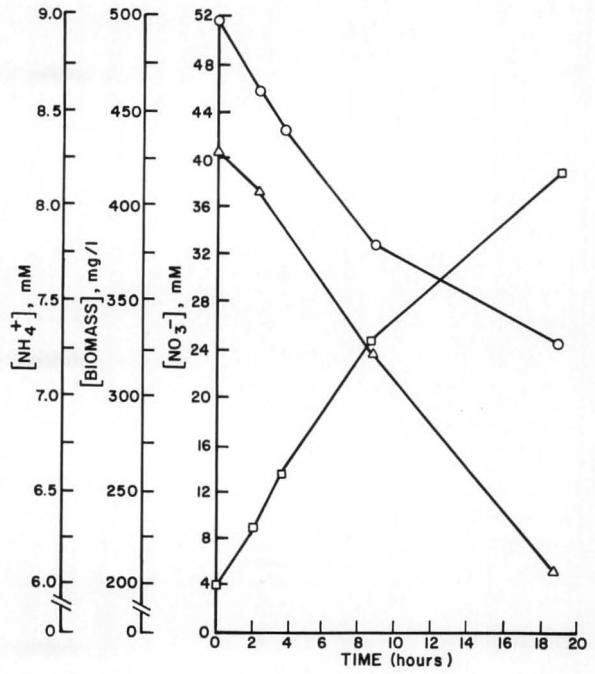

FIGURE 3b. Concentrations of nitrate (NO_3^-), biomass and ammonium (NH_4^+) in an anaerobic* T. denitrificans *batch reactor receiving 1.25 mmoles/hr hydrogen sulfide (H_2S) feed. NO_3^- (O); NH_4^+ (Δ); biomass (□).

is oxidized and the cell population increases. Lastly, hydroxide is steadily consumed as acid is produced by the process.

ACKNOWLEDGEMENT

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REFERENCES

1. Baalsrud, K., and K. S. Baalsrud, "Studies on *Thiobacillus denitrificans*," *Arch. Mikrobiol.*, **20**, 34-62 (1954)
2. Sublette, K. L., and N. D. Sylvester, "Oxidation of Hydrogen Sulfide by *Thiobacillus denitrificans:* Desulfurization of Natural Gas," *Biotech. and Bioeng.*, **29** 249-257 (1987)
3. Sublette, K. L., and N. D. Sylvester, "Oxidation of Hydrogen Sulfide by Continuous Cultures of *Thiobacillus denitrificans*," *Biotech. and Bioeng.*, **29**, 753-758 (1987)
4. Sublette, K. L., and N. D. Sylvester, "Oxidation of Hydrogen Sulfide by Mixed Cultures of *Thiobacillus denitrificans* and Heterotrophs," *Biotech. and Bioeng.*, **29**, 759-761 (1987)
5. Suzuki, I., "Mechanisms of Inorganic Oxidation and Energy Coupling," *Ann. Rev. Micro.*, **28**, 85-101 (1974)
6. American Public Health Association, *Standard Methods for the Examination of Water and Wastewater,* 14th Ed., APHA, New York (1976)
7. Meites, L., Ed., *Handbook of Analytical Chemistry,* McGraw-Hill Book Co., New York (1963)
8. Schedel, M., and H. G. Truper, "Anaerobic Oxidation of Thiosulfate and Elemental Sulfur in *Thiobacillus denitrificans*," *Arch. Mikrobiol.*, **124**, 205-210 (1980)
9. Folin, O., and V. Ciocalteau, "On Tyrosine and Tryptophan Determinations in Proteins," *J. Biol. Chem.*, **73**, 627-649 (1927)
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein Measurement with the Folinphenol Reagent," *J. Biol. Chem.*, **193**, 265-275 (1951) ❐

ChE book reviews

ADVANCES IN DRYING,
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Reviewed by
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The drying of solids is probably one of the oldest unit operations practiced by man. In the past, this process generally was considered to mean the removal of moisture from matter when the amount of same was relatively small. However, in the chemical processing industry of today, feedstocks to be dried may contain as much as ninety percent moisture, and that moisture many times may be nonaqueous and multicomponent. Moisture removal can be effected by (i) condensed-phase separation, (ii) chemical decomposition, (iii) chemical precipitation, (iv) absorption, (v) adsorption, (vi) expression and (vii) vaporization. Convection drying, *i.e.*, moisture removal by vaporization with the drying medium being both the energy source and moisture sink, is by far the most common method used. The materials-handling considerations resulting from many different feedstock and product requirements inevitably resulted in the development of many types of equipment—each with its own operation idiosyncrasies.

In recent years, the number of monographs, handbooks, journals, proceedings and research reports devoted to this subject has increased markedly with the series entitled *Advances in Drying* which was initiated in 1980. In the preface of Volume 4, the editor indicates that this ". . . series is designed to allow individuals concerned with (various) aspects of drying to access relevant information in a carefully reviewed form with minimal time and effort." Like its predecessors, this work consists of a number of reviews, updates, and developments concerning theory, design, and practice in connection with moisture transfer through and/or removal from solids. Eight individual topics are addressed from various viewpoints by contributors from seven countries.

Computer-aided design of convection dryers is discussed in Chapter 1. The classification of mathematical models according to contact zones and flow patterns, the systematic application of the overall mass and energy balances, and the simplification of drying mechanisms is presented. Most of the chapter is devoted to examples of recommended calculation procedures for different types of dryers with the coverage of spray and rotary dryers being especially minimal. Chapter 2 deals with recent advances in the drying of wood. A review of drying theory and modeling is followed by a good summary of recent developments in lumber and veneer drying. Recommendations for future work are presented. Chapter 3 contains a condensed theoretical review of the drying of porous solids with stress on the internal mechanism of moisture and energy transfer. Coupled heat and moisture transfer in soil is reviewed in Chapter 4. Written from

Continued on page 43.